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# Wheat as a dual crop for biorefining: Straw quality parameters and their interactions with nitrogen supply in modern elite cultivars

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## Abstract

Agricultural residues, such as straw, offer an opportunity to produce biofuels and chemicals in biorefineries without compromising food production. The ideal “dual-purpose cultivar” would have high yield of grain and straw. In addition, the straw should be easy to process in a biorefinery: It should have good degradability, high concentration of carbohydrates, and low concentration of ash. Nitrogen (N) is an essential nutrient important for plant growth, crop yield and grain quality. However, N production and application comes with a high cost and high environmental footprint. The N application should consequently be based on an economical optimum. Limited knowledge exists on how N application affects the potential of straw for biorefining, *for example*, straw yield and quality. This study, conducted over three cropping seasons, investigated the effect of N supply on the biorefining potential and included 14 wheat cultivars and one triticale cultivar. The N supply directly affected the yield of straw and grain. In addition, the protein concentration in grain and straw increased, but the composition of the straw with respect to carbohydrates and lignin was largely unaffected by N supply. The only significant change was a lower silicon concentration at increasing N application rate, which could be beneficial for lignin valorization in biorefineries. Likely due to the negligible changes in cell wall composition, the effect of N application rate on straw degradability was not significant. N application should therefore primarily be optimized with respect to grain quality and overall yield of grain and straw. Differences between cultivars were also minor with respect to their performance in a biorefinery process. From a breeding and agronomic perspective, focus should therefore be put on maximizing the biomass output from the field, *that is*, selecting the cultivar with highest grain and straw yield and optimizing the application of fertilizer to get optimum N use efficiency.

## KEYWORDS

biofuels, cell wall composition, fertilizer application, grain yield, silicon, straw degradability, straw yield, triticale

## 1 | INTRODUCTION

Globally, wheat is with around 740 million tonnes the second most produced cereal and the number one cereal in terms of human consumption (Food & Agriculture Organization of the United Nations, 2017). Due to the major importance of wheat for human nutrition, breeding programs have traditionally focused on seeking short-statured, lodging- and pathogen-resistant, high-yielding, nitrogen (N)-responsive wheat cultivars (Lammerts van Bueren & Struik, 2017; Townsend, Sparkes, & Wilson, 2017). Little to no emphasis was given on the production of straw as a valuable commodity since traditional applications of wheat straw include, among others, plowing it back to the soil to maintain soil organic matter, forage for ruminants, animal bedding, and burning for heat and electricity production. However, wheat straw is gaining more interest as a feedstock for biorefineries for the production of second-generation fuels and chemicals (Larsen, Haven, & Thirup, 2012; Parajuli et al., 2015). In Denmark, the first demonstration-scale facility for production of bioethanol from wheat straw was inaugurated in 2009 (Larsen, Haven, et al., 2012). Beta Renewables from Italy started commercial production of bioethanol in 2013 (Johansen, 2016), and in 2017, the company Clariant announced the planned construction of a commercial bioethanol plant based on wheat straw in Romania. The fact that wheat can provide both grain for food/feed and straw for biorefining makes it a potential ideotype for a dual-purpose cultivar (DPC) (Townsend et al., 2017).

The global availability of wheat straw for biorefineries is difficult to assess as straw yields are rarely reported and influenced by several factors, such as water and N availability, sowing rate and date, and cultivar selection (Barraclough et al., 2010; Dai et al., 2016; Donaldson, Schillinger, & Dofing, 2001; Muhammad et al., 1996; Townsend et al., 2017). The amount of globally produced wheat straw can be estimated based on wheat grain yield. Taking the aforementioned wheat production and assuming an average harvest index (HI) of 0.57 (this study), approximately 560 Mt of straw is produced. A previous study estimated a wheat straw residue production of 800 Mt (Bentsen, Felby, & Thorsen, 2014). The amount of straw that is potentially available for biorefining will also depend on collection efficiency, feasibility of transport, and the willingness of farmers to actually sell straw (Townsend et al., 2017).

Given the growing interest in exploiting the potential of straw for biorefining, there is a demand to develop wheat cultivars with improved characteristics as DPC. Previous breeding work has focused on improving HI in part by incorporating dwarfing genes and thereby redirecting nutrients and energy toward higher proportion of grain. As an

example, modern cultivars have at least a 50% increase in grain yield compared to historical cultivars without a concomitant increase in total aboveground biomass (Austin, Ford, & Morgan, 1989). The improvements in grain yield observed within the last two to three decades have thus not followed this trend (Shearman, Sylvester-Bradley, Scotte, & Foulkes, 2005), likely because HI has approached the theoretical limit. Future increases in grain yield will hence depend on increasing total aboveground biomass, *that is*, more leaf and stem (Gaju et al., 2016). This development is well in line with the concept of developing new DPC ideotypes to meet the demand for straw for biorefining.

In a biochemical biorefinery, the sugars making up the polysaccharides embedded in the plant cell wall first have to be released. The overall potential for biorefining, *for example*, sugar production or ultimately ethanol production, is thus a combination of biomass yield, carbohydrate content, and how efficiently these can be released as monosaccharides within a given process, *that is*, the saccharification efficiency (Escamez et al., 2017). Due to the inherent recalcitrance of lignocellulosic biomasses, such as wheat straw, a pretreatment step is required before enzymes can be used to perform the saccharification (Brethauer & Studer, 2015; Jørgensen, Kristensen, & Felby, 2007). Several pretreatment technologies exist, but for agricultural residues such as wheat straw, hydrothermal pretreatment has successfully been applied and operated in demonstration scale (Larsen, Haven, et al., 2012). Biomass recalcitrance is associated with several factors, including lignin composition and concentrations (Himmel et al., 2007), which correlate with reduced sugar release for several biomasses (Chen & Dixon, 2007; Fu et al., 2011). Other factors such as cellulose crystallinity as well as content and type of hemicellulose including possible cross-linking to lignin also play a role during saccharification (McCann & Carpita, 2015). Secondary factors such as concentration of silicon, which is involved in mechanical strength and biotic protection of the plant (Le, Sørensen, Knudsen, & Meyer, 2015), have also been speculated to be involved in recalcitrance of the biomass (Murozuka et al., 2014). Due to this complexity of numerous factors interacting, it is difficult to predict the recalcitrance of a certain biomass and accordingly do directed engineering to get less recalcitrant plants (Djajadi et al., 2017; Merali et al., 2016). In recent years, one approach has therefore been to do large screening studies in order to take advantage of the natural variation in order to select and breed for cultivars with better traits for bioconversion (Bellucci et al., 2015; Collins et al., 2017; Lindedam et al., 2012; Selig et al., 2010). In the context of developing an ideal DPC, it is important to select cultivars giving high biomass yield without compromising grain yield.

The application of the macronutrient N is crucial for grain yield and quality, in particular protein content. Little

attention has been paid to how straw yield and composition are influenced by the rate of N application, and the information available is inconsistent (Townsend et al., 2017). Varying N application has been shown to alter lignin concentration in wheat straw (Murozuka et al., 2014), and possibly also the cell wall carbohydrate composition (Baldwin et al., 2017). The actual effect on saccharification was however negligible for one tested cultivar (Baldwin et al., 2017). The limitation of these previous studies was that they either analyzed the effect of N application on biomass production for one or few cultivars (Barraclough et al., 2010; Murozuka et al., 2014) or tested the effects of several cultivars on sugar release potential without changes in N application (Larsen, Bruun, & Lindedam, 2012). There is, thus, a lack of field data collected across several locations and/or seasons to increase the understanding of how the rate of N application affects the straw yield of different wheat genotypes and the consequences for the quality of the straw for biofuel and biorefining.

The aim of the present study was to increase the understanding of how the rate of N application affects the yield and quality of wheat straw for biofuel/biorefining. The study involved 14 commercial winter wheat cultivars, with morphological distinct features covering a range of potential straw yields. In addition, one triticale cultivar was included. The 15 cultivars were grown on different fields at the experimental farm of the University of Copenhagen for three cropping seasons to account for genotype and environment interactions. The influence on yield and quality was studied by measuring biomass production (straw and grain), grain protein content, straw cell wall composition, and the potential sugar release from straw.

## 2 | MATERIALS AND METHODS

### 2.1 | Study sites, experimental design, and plant material

Field experiments were conducted during three seasons (2012/2013, 2013/2014, and 2014/2015) at different fields belonging to the experimental farm of the University of Copenhagen in Taastrup (Denmark). The 2012/2013 and 2013/2014 field experiments were performed on fine sandy clay soil, whereas the 2014/2015 experiment was performed on coarse sandy clay. Sowing took place in mid-September in 2013 and 2014, but was delayed until early October in 2012 due to unfavorable weather conditions (excessive rainfall).

The climatic conditions were overall temperate and wet, with mild summers and cool to cold winters. Briefly, the 2012/2013 growth season was characterized by a cold and dry spring (March–April) with negative effects on crop growth, followed by a very wet May and a long drought

period in July and August. Growth conditions were better in the 2013/2014 season. Here, the winter and spring period was relatively warm with ample rainfall. Temperatures were also relatively high in June and July 2014, while rainfall was below average. During the 2014/2015 season, conditions were optimum with ample rainfall throughout the period and normal temperatures. The previous crops on the fields were for growing season 2012/2013 spring barley and spring barley (pre-precrop and precrop), 2013/2014 spring barley and oats, and 2014/2015 spring barley and spring barley. In all years, the straw from the precrops had been incorporated into the soil. At the experimental site used in 2013/2014, a nitrogen catch crop consisting of oil radish (*Raphanus sativus* L. var. *oleiformis* Pers) had been incorporated into the soil in spring 2012, while the site used in 2014/2015 had been cultivated with red fescue (*Festuca rubra*) in 2011.

The 14 cultivars of commercialized winter wheat (*Triticum aestivum* L.) and one triticale ( $\times$  *Triticosecale*) were selected primarily to obtain a variation in straw length and related straw parameters (Table 1). Three levels of N fertilizer, viz., 100, 160, or 220 kg N ha<sup>-1</sup>, were applied to 36 m<sup>2</sup> large randomized plots in three blocks. The N was split between 50 kg/ha in mid-March and 50, 110, or 170 kg N ha<sup>-1</sup> in mid-May. Plants were additionally fertilized with approx. 30 kg/ha of P and 75 kg/ha of K in mid-March. Fields were managed according to requirements with weed control and fungicide application. At maturity, plants were combine-harvested using a Haldrup experimental harvester (Løgstør, Denmark) and the grain and straw weighed. The stubble height was 14 cm, which can be considered representative of normal harvest. Straw was collected directly into large bags with minimal loss of leaves and chaff. Representative subsamples were taken, weighed, and dried to constant weight at 60°C. Before analysis, samples were finely ground by use of a ball mill.

### 2.2 | Carbon and N concentration

Carbon and N in the grain and straw material were analyzed based on the Dumas dry-combustion method in an ANCA-SL Elemental Analyser coupled to a 20–20 Tracer-mass Mass Spectrometer (SerCon Ltd., Crewe, UK) according to Murozuka et al. (2014). Crude protein (% w/w) was calculated from N (% w/w) using a conversion factor of 6.25.

### 2.3 | Straw mineral composition

Pulverized plant material (100 mg) was weighed into Teflon microwave digestion tubes followed by addition of 2.5 ml of 70% (v/v) nitric acid. The tubes were then capped, and the samples were digested in a microwave

**TABLE 1** List of cultivars used in the field experiment

Genotype	Crop	Breeder	Released	Characteristics
Ambition	Winter wheat	Nordic Seed	2005	Medium long straw, low culm density
Audi	Winter wheat	Nordic Seed	2006	Long straw, low culm density
Creator	Winter wheat	Sejet	2013	Bushy, grass-like type
Evolution	Winter wheat	Sejet	2010	English type with low straw yield
Genius	Winter wheat	NordSaat		Relatively long, strong straw; bread wheat
Gedser	Winter wheat	Nordic Seed	2011	Short straw
Hereford	Winter wheat	Sejet	2007	Well adapted, medium straw yield
Hybery	Winter wheat	Saaten-Union		Hybrid wheat with long stiff straw
JB Asano	Winter wheat	Breun		High straw yield, long straw
Jensen	Winter wheat	Nordic Seed	2009	Long straw
Mariboss	Winter wheat	Nordic Seed	2008	High straw yield, high culm density
Nakskov	Winter wheat	Nordic Seed	2012	Short straw
Pierrot	Winter wheat	Sejet	2009	Long straw
Trilobit	Triticale	Sejet	2012	High straw yield
Tuareg	Winter wheat	NordSaat	2007	Long straw

oven at 242°C for 25 min (UltraWAVE single-reaction chamber microwave digestion system, Milestone Inc., Shelton, CT; Multiwave 3000, software version 1.24, Anton Paar GmbH, Graz, Austria). After the microwave digestion, 0.2 ml of 49% (v/v) hydrofluoric acid and 2 ml of 36% (v/v) hydrochloric acid were added and the samples were incubated at room temperature overnight. The digested samples were then diluted with Milli-Q water up to a volume of 50 ml. The elemental composition of the sample was measured by inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 5300 DV; PerkinElmer, Waltham, MA). Reference material (spinach leaf, NCS ZC73013; China National Analysis Center for Iron and Steel, Beijing, China) was included in the analysis.

## 2.4 | Cell wall extraction

Samples enriched in plant cell wall polymers (alcohol-insoluble residue, AIR) were prepared according to Baldwin et al. (2014). Briefly, ground plant material (100 mg) was washed twice in 96% ethanol at 70°C. The extract was homogenized in 50 mM Tris-HCl (pH 7.2) containing 1% (w/v) SDS detergent at 70°C for 30 min. Cell walls from the homogenate were collected on a nylon mesh filter disk (Millipore, 41 µm) positioned in a Millipore-type manifold, washed sequentially with plenty of water, ethanol, and acetone, and then resuspended in water. Finally, the material was freeze-dried. Dry cell wall material was digested with  $\alpha$ -amylase. Briefly, the cell walls were suspended in 100 mM potassium phosphate (pH 6.8) and digested for 24 hr at room temperature with  $\alpha$ -amylase (0.51 U of  $\alpha$ -amylase from *Bacillus subtilis* type II-A). After digestion,

the cell walls were centrifuged at 3000 g for 5 min, washed three times with water, and freeze-dried.

## 2.5 | Crystalline cellulose concentration

Cell wall sugars other than cellulose were hydrolyzed using 2 N trifluoroacetic acid for 90 min at 121°C. The supernatant was discarded, and the pellet was washed several times with water and dried. The cellulose concentration was measured according to the method based on material resistant to acetic nitric hydrolysis (Domon et al., 2013).

## 2.6 | Lignin concentration

Lignin concentration was determined using the acetyl bromide method (Domon et al., 2013). Dry cell walls (50 mg) were incubated with 2.5 ml of acetyl bromide reagent (25% (v/v) acetyl bromide in glacial acetic acid) for 4 hr at 50°C. After cooling to room temperature, 1.5 ml of the reaction mixture was spun at 13,400 g for 3 min. To 500 µl of clarified supernatant, 2 M NaOH (2 ml) and acetic acid (2.4 ml) were added. Hydroxylamine (350 µl) was added to each sample and diluted to 10 ml with acetic acid. Absorbance was read at 280 nm. The amount of lignin was calculated using the extinction coefficient that was assumed to be 17.688 L g<sup>-1</sup> cm<sup>-1</sup>.

## 2.7 | Enzymatic saccharification

Pretreatment and enzymatic saccharification were performed following the method described by Zhang et al. (2014). Briefly, 25 mg of the dried plant material was weighed into a 96-well aluminum plate. 50 mM sodium



citrate buffer (pH 5.0) was added to the samples, and the plate was sealed with Teflon tape with a little hole above each well. A thin aluminum plate, a Teflon plate, and a thick aluminum plate were placed on top of the 96-well aluminum plate, and they were heated in a custom-built heating block with a stable pressure at 190°C for 10 min. After cooling the plate to room temperature, the enzymatic saccharification was performed by adding directly in the plate 50 µl of the commercial cellulase preparation Cellic CTec2 (Novozymes, Bagsværd, Denmark) diluted 17 times by weight with sodium citrate buffer (pH 5.0) to each sample. This enzyme loading corresponded to 5.0 filter paper unit (FPU) per gram dry matter (g/DM). The plate was incubated in a plate shaker at 50°C at 600 rpm for 72 hr. The hydrolyzed samples were then filtered through 0.45 µm filter plates (Pall Corporation, Ann Arbor, MI). 100 µl of each filtrate was mixed with 100 µl of 5 mM sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) for sugar measurements using high-performance liquid chromatography (HPLC). Separation and determination of the glucose and xylose in the filtrates were carried out by Ultimate 3000 HPLC (Dionex, Germering, Germany) equipped with a refractive index detector (Shodex, Tokyo, Japan). The separation was performed in a Phenomenex Rezex ROA column (Phenomenex Inc., Torrance, CA) at 80°C with 5 mM H<sub>2</sub>SO<sub>4</sub> as eluent at a flow rate of 0.6 ml/min. The same wheat straw reference material (NIST 8494) was included in all plates and years to normalize the results for batch variations.

## 2.8 | Statistical methods

R (version 3.2.1) was used for all statistical tests. Trait data were adjusted for outliers (<2% of data either missing or with errors in the recording) and modeled by linear models (lm in R), for each year separately (Equation (1)) and for combined years (Equation (2)).

$$Y = \mu + B_k + N_j + C_l + NC_{jl} + \varepsilon \quad (1)$$

$$Y = \mu + A_i + B_{ik} + N_j + C_l + NC_{jl} + \varepsilon \quad (2)$$

$Y$  denotes the dependent variable that corresponds to trait values (grain yield, straw yield, grain  $N$  content, harvest index, cellulose concentration, lignin concentration, silicon concentration, glucose and xylose release, sugar potential).  $\mu$  is the overall mean, while the other parameters are the explanatory variables that define the additive effect of year ( $A_i$ , where  $i = 2013\text{--}2015$ ), block ( $B_{ik}$ ,  $k = 1\text{--}3$ ),  $N$  level ( $N_j$ ,  $j = 100\text{ N}, 160\text{ N}, 220\text{ N}$ ), cultivar ( $C_l$ ,  $l = 1\text{--}15$ ), and the interaction between cultivar and  $N$  level ( $NC_{jl}$ ). Finally,  $\varepsilon$  defines the residuals. Multiway ANOVA was used to fit the models excluding nonsignificant independent variables and to determine the effect of cultivar, nitrogen level, and/or interaction. Tukey's honestly significant difference

(HSD) test (HSD.test in R) was used for pairwise comparison between nitrogen levels or cultivars.  $N$  or cultivar effects were considered significant for the particular trait if the  $p$ -value were below 0.05.

## 3 | RESULTS

### 3.1 | Straw and grain yields

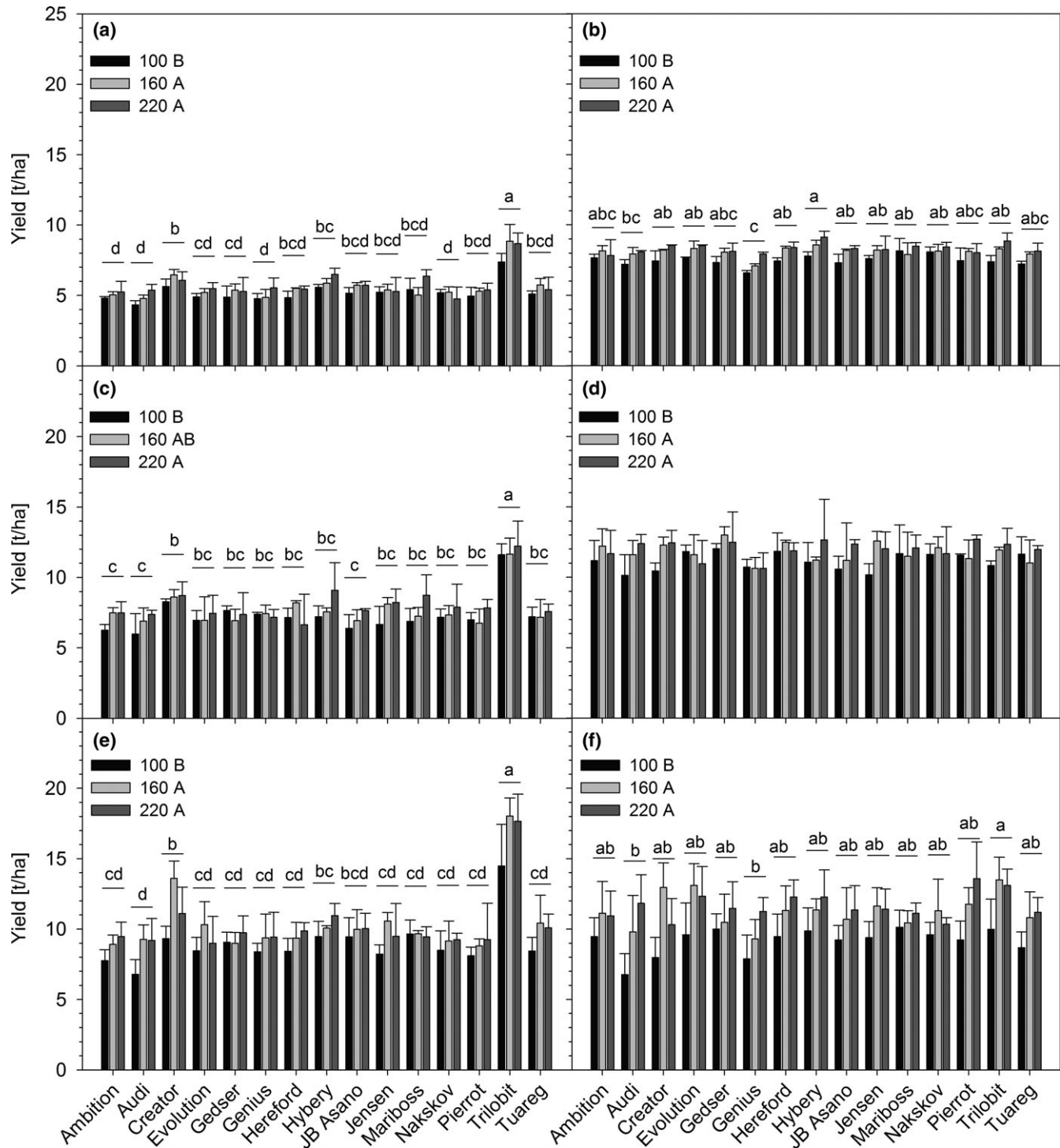
The 15 cultivars were harvested at maturity in a field experiment carried out over three consecutive growing seasons (2012/2013, 2013/2014, and 2014/2015). Major differences in straw yields were observed between years ( $p < 0.001$ ; Figure 1a,c,e; see also Data S1). Straw yields were on average 5.5, 7.7, and 9.9 t/ha in 2013, 2014, and 2015, respectively. The grain yields were on average 8.0, 11.7, and 10.7 t/ha in 2013, 2014, and 2015, respectively ( $p < 0.001$  for differences between years; Figure 1b,d,f; see also Data S1). The relatively low yields in 2013 might be attributed to the 2012/2013 field experiment being affected by relatively late time of sowing, wet seedbed, and drought spells during spring and summer. In the following two years, the experiments were better established in a significantly more optimal seedbed.

There was a significant effect of  $N$  application on straw and grain yields ( $p < 0.001$  for both). Straw and grain yields were significantly higher (11%–16% and 8%–28%, respectively) at the highest rate of  $N$  (220 kg/ha) compared to the lowest (100 kg/ha). Application of 160 kg  $N\ ha^{-1}$  relative to 100 kg  $N\ ha^{-1}$  significantly increased straw yields (8% to 16%) in 2013 and 2015 as well as grain yields (6%–23%) in all three years. No significant differences in straw yields were observed between 160 and 220 kg/ha in all three years.

Genotypic differences in straw yield were observed ( $p < 0.001$ ; Figure 1a,c,e; Data S1). Trilobit, which is a triticale cultivar (hybrid between wheat and rye), had the significantly highest straw yield in all three years, 50% to 70% higher than the average yield. Creator, a winter wheat cultivar characterized as a bushy type (Table 1), had also significantly higher straw yield for the three years (10% to 14% higher than average yield). Some cultivars had significantly lower straw yields. Audi and Ambition showed 13%–16% and 8%–12% lower straw yields than the average yield, respectively.

Grain yields also differed significantly among cultivars ( $p < 0.001$ ). No cultivar yielded consistently higher grain yields in all three years (Figure 1b,d,f; Data S1), but the bread wheat genotype Genius had in each of the years about 10% lower yield than the overall average, which was 10.1 t/ha. On average, Trilobit had the highest grain yield (10.7 t/ha). Thus, the higher straw yield of this genotype did not compromise grain yields.

Based on the grain and straw yield, the average HI was 0.57.  $N$  application had no impact on the HI for



**FIGURE 1** Straw (a, c, d) and grain (b, d, f) dry matter yields of 15 cultivars. Samples were harvested in 2013 (a, b), 2014 (c, d), and 2015 (e, f) at maturity after application of 100, 160, and 220 kg N ha<sup>-1</sup> in spring. Values are means  $\pm$  S.E. ( $n = 3$ ). Data were analyzed with ANOVA followed by a Tukey HSD test. Different letters denote significant difference at  $p = 0.05$ . Capital letters after figure legends represent differences due to N effect, and lowercase letters above columns differences due to cultivar effect. For grain yield, there were no significant differences between cultivars in 2014

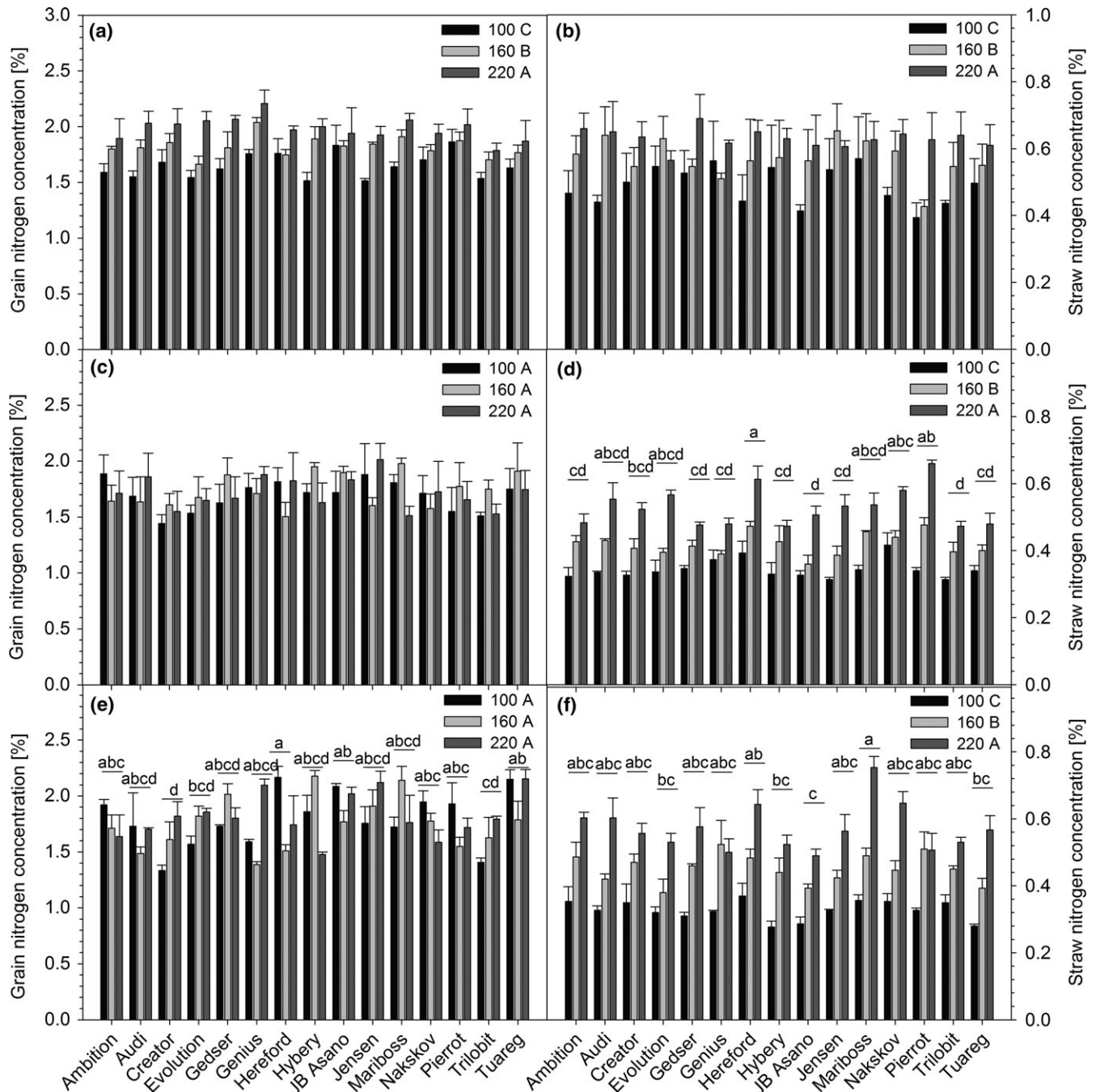
both 2013 and 2014. However, in 2015, intermediate and high N applications (160 and 220 kg/ha) resulted in significantly higher HI values than the low N rate (100 kg/ha). No cultivar showed a consistently higher

HI throughout all three years. However, HI was all three years significantly lower for Trilobit (15%–20% lower than average). HI of Creator was also below average (3%–8%).

### 3.2 | Grain protein and straw N concentration

Despite rather similar grain yields across the tested cultivars, the grain N concentration was statistically different among cultivars when analyzed for all three years

( $p < 0.001$ ; Figure 2; Data S1). Triticale had the lowest grain N concentration, on average 1.63% corresponding to 10.2% crude protein. JB Asano had the highest grain N concentration, on average 1.88% corresponding to 11.75% crude protein. On average, the N concentration was 1.71% at 100 kg N ha<sup>-1</sup> and 1.84% at 220 kg N ha<sup>-1</sup>



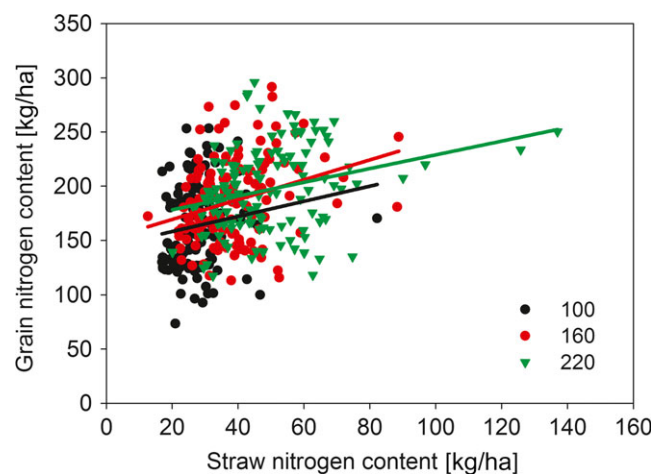
**FIGURE 2** Grain nitrogen concentration (a, c, e) and straw nitrogen concentration (b, d, f) of 15 cultivars. Samples were harvested in 2013 (a, b), 2014 (c, d), and 2015 (e, f) at maturity after application of 100, 160, and 220 kg N ha<sup>-1</sup> in spring. Values are means  $\pm$  S.E. ( $n = 3$ ). Data were analyzed with ANOVA followed by a Tukey HSD test. Different letters denote significant difference at  $p = 0.05$ . Capital letters after figure legends represent significant differences due to N effect, and lowercase letters above columns represent differences due to cultivar effect. For grain N concentration, no significant difference between cultivars was observed in 2013 and 2014. For straw N concentration, there was no significant effect of cultivar in 2013



and with a significant difference for all years ( $p < 0.001$ ). When analyzed for each year, a significant effect of the rate of N application was only seen for the 2013 harvest.

The straw N concentration was significantly influenced by N application ( $p < 0.001$ ; Figure 2; S1). The average N concentration was 0.39%, 0.48%, and 0.58% at N application rates of 100 kg/ha, 160 kg/ha, and 220 kg/ha, respectively. There were significant differences in straw N concentration among the cultivars ( $p = 0.01$ ), with Mariboss having the highest average concentration (0.53%) and JB Asano the lowest (0.44%) (Data S1).

Comparing grain N concentration versus straw N concentration at the three N application rates, it was apparent that increasing the N application rate had the most pronounced effect on straw N concentration (Figure 2). However, there was not a clear trend that low grain N concentration was correlated with high straw N concentration or vice versa. Taking into account the yield of straw and grain, *that is*, estimating the N content (total amount of N accumulated per ha in grain or straw), the response to N application rate was more distinct for grain (Figure 3). The N content in the grain corresponded to up to 250 kg/ha at application of 100 kg/ha of N and up to 300 kg/ha at 220 kg/ha of N, thus showing a considerable contribution of N originating from mineralization of soil organic matter. The N content of the straw was between 20 and 140 kg/ha. The highest straw N content at each N application rate was for Trilobit due to the higher straw yield compared to other cultivars. In accordance with this, the N harvest index (NHI) was significantly lower for Trilobit (on average



**FIGURE 3** Straw N content (kg/ha) versus grain N content (kg/ha). Black, red, and green dots represent sample pairs from 100, 160, and 220 kg/ha N application rate, respectively. Solid line is linear fit of data for each of the three N application rates. The slopes of the linear fit were different from zero ( $p$ -values were 0.09, 0.0007, and 0.0012 for N application rates of 100, 160, and 220 kg/ha N, respectively).

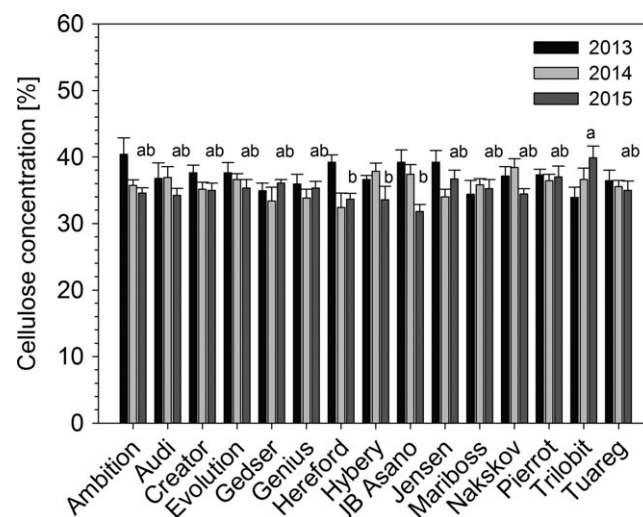
0.76). For the remaining cultivars, there was no difference in NHI. The overall average NHI was 0.83. NHI decreased with increasing N application rate (0.85 at 100 kg/ha, 0.83 at 160 kg/ha, and 0.80 at 220 kg/ha).

### 3.3 | Straw cell wall composition

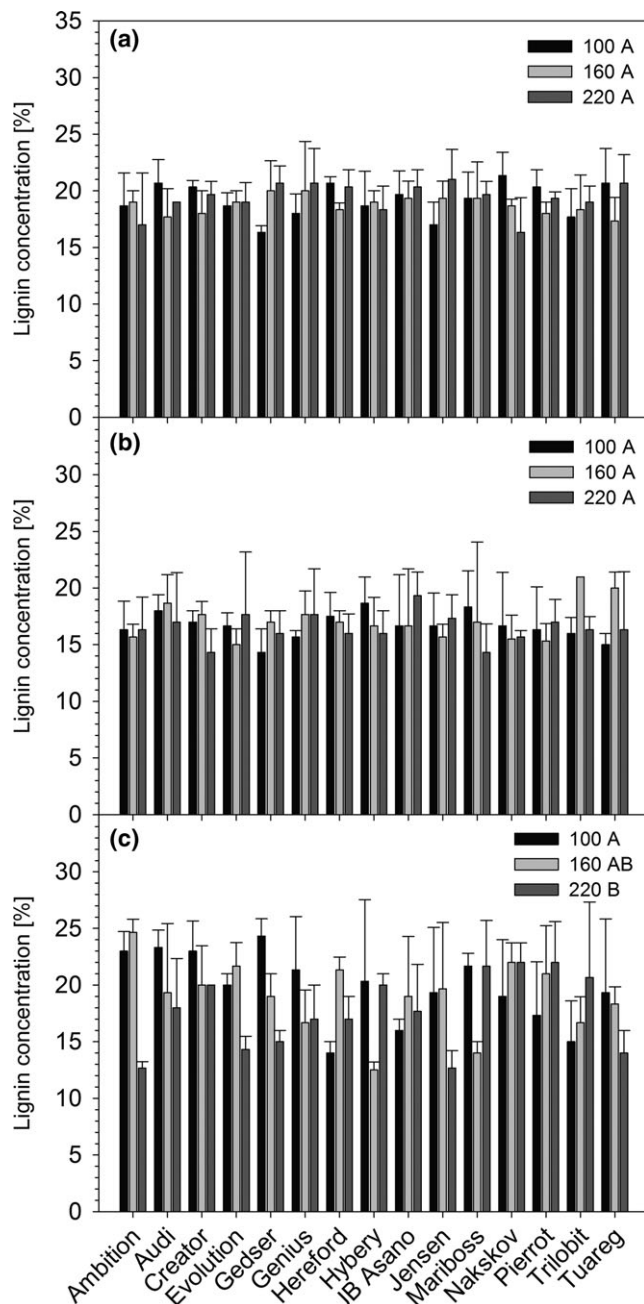
The concentration of the cell wall components cellulose and lignin was analyzed together with the concentration of Si in the straw of the 15 cultivars for all three years (Figures 4, 5, and 6).

The cellulose concentration was not significantly different among the cultivars ( $p = 0.22$ ) and also not influenced by the N application ( $p = 0.23$ ) when analyzed across the three experimental years. Only in 2015, there was a significant effect of cultivar ( $p = 0.0167$ ), and the cellulose concentration in Trilobit was significantly higher compared to Hereford, Hybery, and JB Asano (Figure 4c). On average, the cellulose concentration was  $36\% \pm 4\%$ .

Lignin concentrations were measured by quantification of cell wall phenolic groups using the acetyl bromide method. Similar to the cellulose concentration, the lignin concentration was not different among cultivars ( $p = 0.24$ ) and also not influenced by N application ( $p = 0.19$ ) when analyzed for all three years (Figure 5). On average, the lignin concentration was  $18\% \pm 3\%$ . Considering only the 2015 data, the lignin concentration was significantly lower



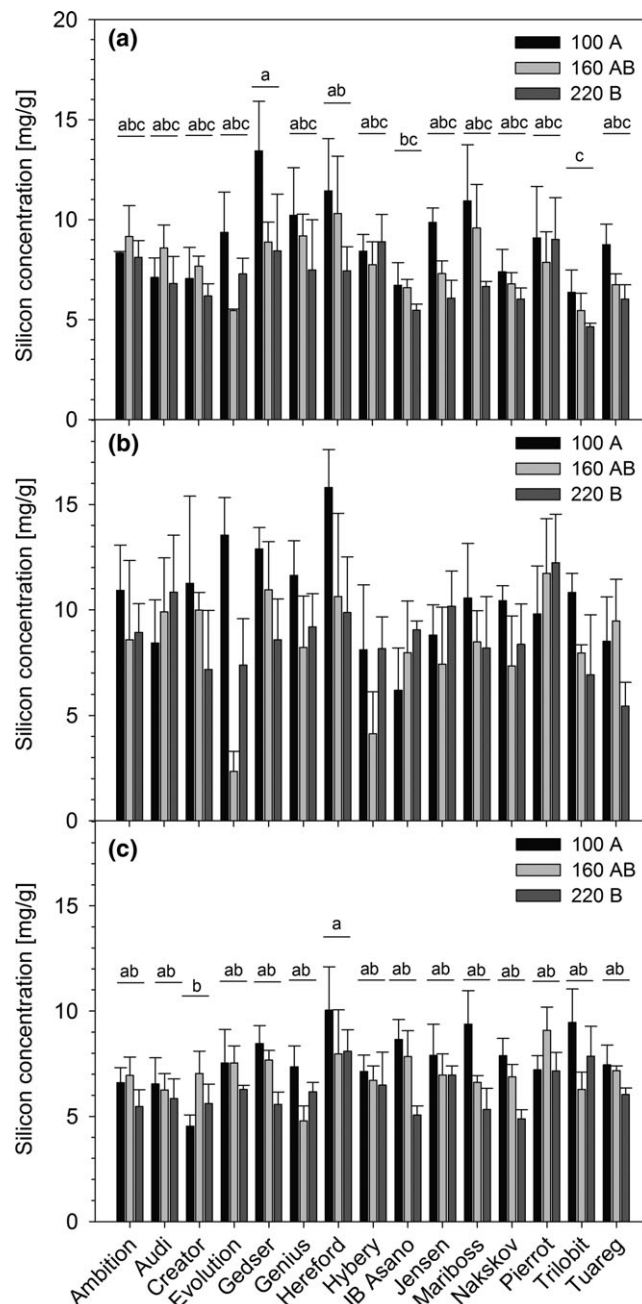
**FIGURE 4** Straw cellulose concentrations of 15 cultivars. Samples were harvested during 3 years at maturity after application of 100, 160, and 220 kg N ha<sup>-1</sup> in spring. Values are means  $\pm$  S.E. ( $n = 9$ ). Data were analyzed with ANOVA followed by a Tukey HSD test. There was no significant effect of N application in any year and no significant effect of cultivars in 2013 and 2014. Different letters denote significant difference at  $p = 0.05$  between cultivars in 2015



**FIGURE 5** Straw lignin concentrations of 15 cultivars. Samples were harvested during 3 years at maturity after application of 100, 160, and 220 kg N ha<sup>-1</sup> in spring. Values are means  $\pm$  S.E. ( $n = 3$ ). Data were analyzed with ANOVA followed by a Tukey HSD test. Different letters denote significant difference at  $p = 0.05$ . Capital letters after figure legends represent differences due to N effect. No significant difference was observed between the cultivars in any of the three years

(11% decrease) at the highest rate of N application (220 kg/ha) compared to the lowest application rate.

Overall for all three years, there was a significant difference in Si concentration among cultivars ( $p = 0.001$ ) and between N application levels ( $p < 0.001$ ). The concentration of Si in the straw from the 15 cultivars correlated



**FIGURE 6** Straw silicon concentrations of 15 cultivars. Samples were harvested in 2013 (a), 2014 (b), and 2015 (c) at maturity after application of 100, 160, and 220 kg N ha<sup>-1</sup> in spring. Values are means  $\pm$  S.E. ( $n = 3$ ). Data were analyzed with ANOVA followed by a Tukey HSD test. Different letters denote significant difference at  $p = 0.05$ . Capital letters after figure legends represent differences due to N effect, and lowercase letters above columns represent differences due to cultivar effect. No significant effect of cultivar in 2014

negatively with N application (Figure 6). At the lowest N application rate of 100 kg/ha, the average Si concentration was significantly higher (9.0 mg/g) compared to that at 160 kg/ha and 220 kg/ha (7.8 mg Si g<sup>-1</sup> and 7.3 mg Si g<sup>-1</sup>, respectively). In 2013, the Si concentration was highest in Gedser (average 10.2 mg/g), whereas Trilobit was

significantly lower (5.5 mg/g). In 2015, the concentration was highest in Hereford (8.7 mg/g) and lowest in Creator (5.7 mg/g), whereas no statistical difference was observed among the cultivars in 2014 (Figure 6).

### 3.4 | Sugar release from straw by enzymatic saccharification

In many biorefinery applications, glucose will be the main source of sugar. Efficient release of glucose from cellulose is therefore the primary objective although attention also has to be paid to the total release of sugar (primarily glucose and xylose in the case of wheat straw). The saccharification performance was measured as glucose and xylose release after standard hydrothermal pretreatment followed by enzymatic hydrolysis. The results (Figure 7) revealed that the amount of glucose released per g of DM was not significantly affected by N application rate ( $p = 0.147$ ). Likewise, the results did not show any statistical difference among the tested cultivars ( $p = 0.197$ ). On average, the lowest glucose release was straw from the cultivar Gedser (0.21 g/g) and the highest from Creator (0.23 g/g). There was a statistical difference between years ( $p = 0.0128$ ), and a Tukey HSD test revealed that the glucose release was higher in 2014 (average 0.224 g/g) compared to 2013 (average 0.216 g $^{-1}$ ), but again the differences were small.

The combined release of glucose and xylose per g DM was very consistent with the glucose release data (data not shown). N application was not a significant effect in the

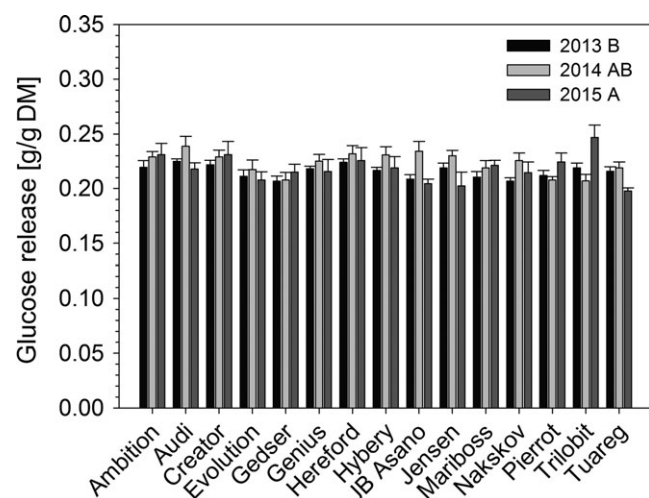
statistical analysis ( $p = 0.56$ ), but cultivar was ( $p = 0.0035$ ). However, a pairwise comparison using the Tukey HSD test did not reveal differences among the cultivars. On average, the combined glucose and xylose yield was  $0.37 \pm 0.03$  g/g. Again, Gedser was the cultivar with the lowest total sugar release (0.35 g/g) and Audi was the highest (0.39 g/g).

Calculating the glucose release relative to the cellulose concentration, *that is*, the cellulose conversion factor, revealed no difference among cultivars ( $p = 0.20$ ) (data not shown). On average, the cellulose conversion factor was  $55\% \pm 9\%$ .

### 3.5 | Sugar potential on field scale

In order to evaluate the true biorefining potential of the cultivars and the effect of N application rate, it is relevant to consider the results on a field-scale basis, *that is*, sugar production potential defined as amount of glucose and xylose that can be produced with this process on a hectare basis. Given the rather small differences among the cultivars with respect to cell wall composition and release of sugar per g DM, it was apparent that straw yield was the main factor responsible for differentiating among the cultivars with respect to sugar production potential (Figure 8; Data S1). Cultivars differed significantly ( $p < 0.001$ ). Trilobit consistently gave the highest sugar production potential (average 5.1 t/ha), which was 38% higher than that of the second best cultivar Creator (3.7 t/ha). The lowest potential was for Pierrot (2.9 t/ha).

N application positively influenced the sugar production potential ( $p < 0.001$ ) (Figure 8; S1). The average sugar production potential among all cultivars was 3.0 t/ha at N application of 100 kg/ha, and it increased to 3.4 g/ha at 220 kg/ha, *that is*, 13% increase.

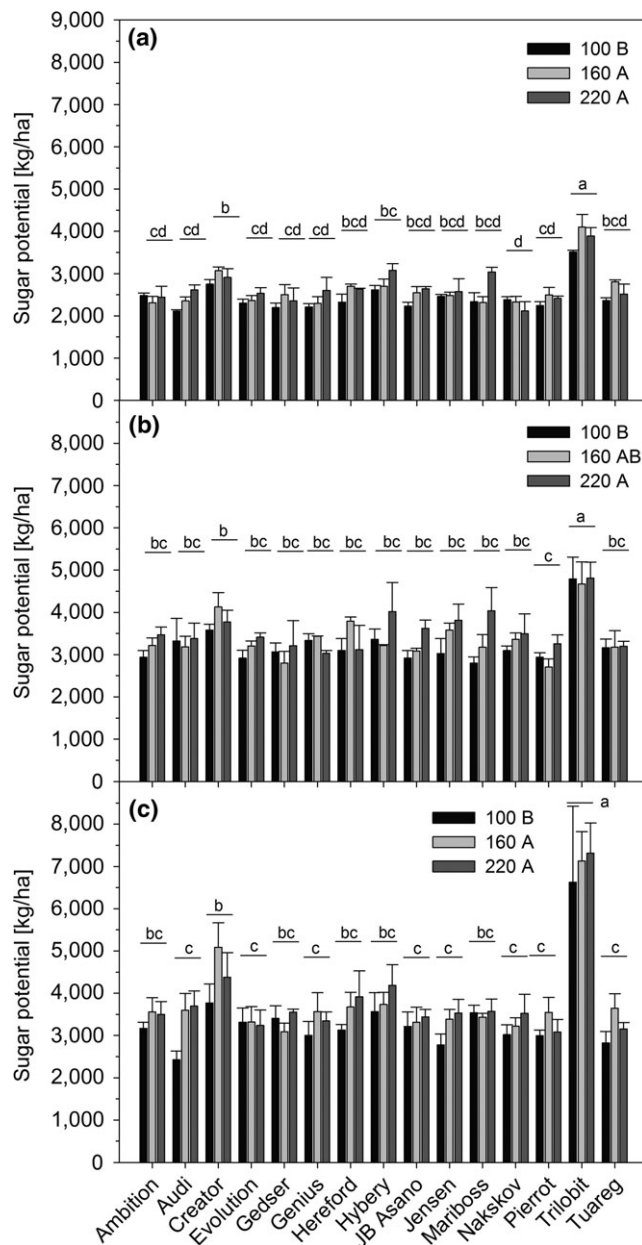


**FIGURE 7** Glucose release per g DM of 15 cultivars after hydrothermal pretreatment and enzymatic hydrolysis. Samples were harvested in 2013, 2014, and 2015 at maturity after application of 100, 160, and 220 kg N ha $^{-1}$  in spring. Data were analyzed with ANOVA followed by a Tukey HSD test. There was no significant effect of N application at  $p = 0.05$ , and no significant difference was observed between the cultivars in any of the three years. Values are means  $\pm$  S.E. ( $n = 9$ )

## 4 | DISCUSSION

### 4.1 | Effect of N fertilization on yield and N concentration of straw and grain

The N application rates used in this study included 100, 160, and 220 kg N per hectare, spanning the rates encountered in commercial wheat production systems without water limitations (Jensen et al., 2011). Whereas the effect of increasing N application rate on grain yield has been extensively studied (Barraclough, Lopez-Bellido, & Hawkesford, 2014; Lu et al., 2015), the number of studies investigating the effect of N application rate on straw yield is fewer (Townsend et al., 2017). In the present work, increased application rate of N from low to either intermediate or high resulted in higher straw yields in all three experimental years. The responsiveness, *that is*, the



**FIGURE 8** Total sugar (glucose and xylose) production potential on a hectare basis. Samples were harvested in 2013 (a), 2014 (b), and 2015 (c) at maturity after application of 100, 160, and 220 kg N ha<sup>-1</sup> in spring. Values are means  $\pm$  S.E. ( $n = 3$ ). Data were analyzed with ANOVA followed by a Tukey HSD test. Different letters denote significant difference at  $p = 0.05$ . Capital letters represent differences due to N effect, and lowercase letters represent differences due to cultivar effect

percentwise increase in straw yield as N application was increased from low to intermediate or low to high, was on average among all cultivars 8% and 12%, respectively (Figure 1a,c,e). Similarly, grain yields increased on average 10% and 15% when N application rate was increased from low to intermediate and from low to high, respectively (Figure 1b,d,f). The results obtained here with these

cultivars tend to show higher response for grain yield as compared to straw yield; however, in other studies, no difference (Lu et al., 2015; Noureldin, Saady, Ashmawy, & Saed, 2013) or the opposite trend has been observed (Gaju et al., 2016). The differences among the studies can be due to the use of different cultivars and not at least also influenced by soil type and the amount of soil N delivered to the crop via mineralization of organic matter. In relation to the use of wheat as DPC, the study documents the possibility to optimize straw and grain yield by modulation of N fertilization.

Among cultivars, there were large differences in responsiveness. This was most pronounced for two cultivars, Nakskov and Pierrot, for which increased N application rate had no or little effect on either grain or straw yield. On the contrary, cultivars such as Creator and especially Audi had high responsiveness for both grain and straw yield. The trend was that the response to N application almost equally affected grain and straw yield. This is in good agreement with other studies that have also documented that N responsiveness on grain and straw yield is often correlated (Gaju et al., 2016). Interestingly, although a large variation in responsiveness (from 4% to 36% for grain) was observed, the grain yields at the highest application rate were not significantly different across cultivars. Similarly, despite large differences in responsiveness, no significant differences in straw yields were observed at the highest N application rate, with the exception of the triticale cultivar Trilobit. In other words, these commercial cultivars performed very similar under optimal N supply, but some cultivars were better at maintaining yield at lower N application rates or had better N uptake efficiency.

During growth, most of the N is found in the form of proteins responsible for the photosynthesis, *that is*, in leaf and stem. At the onset of grain filling, remobilization of proteins from stem and leaves toward the grain starts (Barraclough et al., 2014). Whereas previous studies have documented average increases of grain N concentration in the order of 30% by increasing the N application rate from 100 kg/ha to 200 kg/ha (Barraclough et al., 2010), the average increase observed in this study was very marginal, only 7% when increasing the N application rate from 100 kg/ha to 220 kg/ha (Figure 2). The lower response in this study could be due to the high uptake of soil-derived N originating from mineralization of organic crop residues incorporated in previous years. Interestingly, the effect of increasing N application rate on the straw residual N concentration at maturity was much larger (Figure 2). The N concentration in the straw increased on average 50% when applying 220 kg/ha of N as compared to 100 kg/ha, and there was a significant difference between all three N application rates. It is therefore apparent that the residual N concentration in the straw was much more responsive to N



application rate as compared to grain N. The increasing residual N concentration in the straw represents a loss of potential protein in the grain. From an economic and environmental perspective, it would be preferable if as much as possible N was remobilized to the grain. However, the results did not support that high straw N accumulation occurred at the expense of grain N because a negative correlation between grain N and straw N was not observed neither on the basis of the N concentration per unit dry matter nor on the basis of N content per hectare (Figure 3).

## 4.2 | Effect of N fertilization on straw cell wall composition and recalcitrance

For the purpose of wheat to serve as a DPC, *that is*, using the straw for biorefining, the effect of growth conditions and, in this case, the N application rate on the composition of the plant cell wall is of major interest. The ideal straw for a biorefinery process should have high content of carbohydrates, *for example*, cellulose, and low lignin concentration, as lignin is often associated with poor efficiency and yield in the conversion of cellulose to glucose (Godin et al., 2016). Only a limited number of studies have analyzed the influence of N application rate on lignin and cellulose concentration (Townsend et al., 2017). The average cellulose and lignin concentrations observed in the present work were 36% and 18%, respectively, which are in accordance with literature values (Lindedam et al., 2012; Prasad, Singh, & Joshi, 2007). For all three years, it was consistently found that the cellulose concentration was not statistically influenced by N application rate. With respect to lignin, there was statistically higher lignin concentration in 2015 when comparing the lowest and highest N application rates. However, for 2013 and 2014 no effect of N application was observed. In a previous study with only one cultivar, no effect of N application on either cellulose or lignin concentration could be measured (Baldwin et al., 2017). Murozuka et al. (2014) found that in the range of N application similar to the present study, no difference was observed in lignin concentration, but a very low N application rate ( $48 \text{ kg N ha}^{-1}$ ) resulted in a significantly lower lignin concentration. The systematic testing of several commercial cultivars over three growing seasons and on different soil types as in the present study provides an important and solid dataset showing that plasticity in cellulose and lignin concentration is limited and cannot be altered by changing the N application rate within the span of N applications normally used in crop production.

The direct influence of N application rate on saccharification potential, *that is*, recalcitrance, has also not previously been thoroughly investigated (Townsend et al., 2017). In one study, saccharification potential (release of glucose and xylose per g DM) decreased with increasing N

application rate (Murozuka et al., 2014). Contrary to that study, which was based on only one wheat cultivar, no significant effect of N application rate on the release of either glucose or xylose was found in the present study (Figure 7). It should be remarked that in the study by Murozuka et al. (2014), the span of N application was larger ( $48\text{--}288 \text{ kg/ha}$ ). Glucose release could be influenced by cellulose concentration, but given the very consistent cellulose concentration, the glucose release relative to cellulose concentration, *that is*, cellulose conversion factor, did also not prove any difference in recalcitrance as a consequence of N application rate. The strength of the finding in the present study is that it is based on data from 15 cultivars, three cropping seasons, and different fields. The interaction with other environmental factors and soil type is therefore included in the present assessment.

It has been speculated whether Si is involved in biomass recalcitrance as its function in plant tissues is to increase physical strength and protect against biotic stress, *for example*, protecting the plant from enzymatic degradation during fungal attacks (He et al., 2013; Le et al., 2015). Contrary to this, a previous study found that Si concentration and enzymatic saccharification decreased with increasing N application rate (Murozuka et al., 2014). In a study with 20 wheat cultivars, no correlation between Si concentration and recalcitrance could be established (Murozuka et al., 2015). The effect of Si concentration on recalcitrance has been studied in *Brachypodium distachyon* as a model plant by generating a mutant deficient in Si (Głazowska et al., 2018). Significantly more glucose was released by enzymatic hydrolysis of straw from the mutant, but low Si uptake also influenced the cell wall composition, and the mutant had higher cellulose concentration. The cellulose conversion factor, *that is*, recalcitrance, was therefore not significantly different. Data from the present study also support that Si concentration in straw is not directly linked to recalcitrance. Nevertheless, a high concentration of Si in the lignin fraction resulting after biochemical conversion can be problematic for the valorization of the lignin (Le et al., 2015). In a biorefinery perspective, a low Si concentration is therefore preferable. In that respect, the present study shows that the Si concentration was negatively correlated with N application rate. The finding was evident across all cultivars and for all three years.

## 4.3 | Effect of cultivar on yield and biomass components

Engineering of wheat to produce the ideal DPC appears as an attractive route in order to improve the overall economic revenue of the biorefinery process. Unfortunately, genetic engineering of wheat remains problematic due to technical difficulties as well as the strict regulations surrounding the



use of genetically modified wheat cultivars (Tishler, Samach, Rogachev, Elbaum, & Levy, 2015). Therefore, it is highly relevant to analyze already available wheat and triticale cultivars for their potential as a feedstock for biorefinery purposes without compromising grain yield and quality. These results can then feed into breeding programs to improve wheat as a DPC.

There was a significant effect of cultivar on straw yield. Within the wheat cultivars tested, the yield was 30% higher for the best performing cultivar (Creator) compared to the worst performing cultivar (Audi; Figure 1). However, Trilobit, a triticale cultivar, outperformed all tested wheat cultivars in terms of straw yield (50%–70% higher than average). The variation within grain yields was less pronounced, although significant differences were observed. Among the wheat cultivars, the yield was only 17% higher for the best performing cultivar (Evolution) compared to the worst performing cultivar (Genius), which consistently had the lowest grain yield for all three years. Trilobit had 20% higher grain yield compared to Genius. It is worthwhile to remark that besides Trilobit, there was no wheat cultivar which combined the highest straw yield with the highest grain yield. Evolution with the highest grain yield only had modest straw yield and Creator with the highest straw yield had modest grain yield. Based on total above-ground dry matter biomass, Trilobit was clearly superior with average yield of 25.3 t/ha followed by Creator with 21.0 t/ha. Audi had the lowest total yield of 18.0 t/ha. Compared to a previous field experiment, Audi, Hereford, and JB Asano gave in the present study 56%–67% higher total biomass yield (Larsen, Bruun, et al., 2012), but as shown in Figure 1, yields fluctuated largely between years, and will also depend on soil type and conditions. In addition, in the present study, straw yield was measured by collecting all material directly from the harvester including leaf and chaff, whereas in other studies, *for example*, Larsen, Bruun, et al. (2012), straw yield was based on the amount of baled straw or straw collected from the ground. Compared to studies with other cultivars, similar grain yields in the order of 11 t/ha have been obtained (Barraclough et al., 2010). With an N application rate of 180–200 kg N ha<sup>-1</sup>, Gaju et al. (2016) obtained total above-ground biomass yields of 16 to 21 t/ha for five modern UK wheat cultivars. The results reported here for the wheat cultivars are therefore in line with yields obtained in experiments with other modern cultivars. This again highlights the superior yield of Trilobit, which was also much higher compared to, *for example*, the triticale cultivar Dinaro (11.4 t/ha total biomass; Larsen, Bruun, et al., 2012).

Lignin concentrations were consistent over the experimental period and not influenced by cultivars. A previous study investigating the cell wall composition of five wheat cultivars found no statistical difference in the composition

of cellulose and lignin (Lindedam, Bruun, Jørgensen, Felby, & Magid, 2010). A study including 20 wheat cultivars grown at two sites did also not reveal any significant difference in lignin concentration, which was within a narrow range from 18.2% to 20.9% (Lindedam et al., 2012). The cellulose concentration in that study varied between 34.2% and 40.8%, and a difference among cultivars was detected. However, based on results from the present and also previous studies, the general observation is that these modern cultivars are likely too closely related to reveal clear differences in straw quality.

The Si concentration in the straw was influenced by cultivar. On average, Hereford had the highest Si concentration, which was 44% higher than that of JB Asano, which had the lowest Si concentration. Trilobit with the highest straw yield was among the cultivars with the lowest Si concentration. However, overall there was no correlation between high straw yield and low Si concentration, *that is*, a dilution effect.

#### 4.4 | Saccharification potential of straw

In the present study, the saccharification potential, *that is*, how much sugar (glucose and xylose) can be released from the straw, was evaluated by a high-throughput setup that involved a hydrothermal pretreatment followed by enzymatic hydrolysis (Lindedam et al., 2014; Zhang et al., 2014). Based on the amount of glucose or glucose plus xylose released per g of DM biomass, no significant differences between the cultivars (Figure 7) were observed for glucose release, whereas differences were seen for the combined glucose–xylose release. Overall mean values were  $0.22 \pm 0.02$  g/g DM and  $0.37 \pm 0.03$  g/g DM for glucose and glucose plus xylose, respectively, thus being in line with previous results (Bellucci et al., 2015; Lindedam et al., 2014).

Expressing the saccharification on the basis of percentage of cellulose hydrolyzed (cellulose conversion factor) did not reveal significant differences among cultivars either. Based on overall mean values, Hereford had the highest conversion (on average 59% of glucose released from cellulose), whereas Evolution had the lowest (53%). The triticale Trilobit was with 56% conversion just above the overall average of 55%. Previous studies have, to a variable degree, been able to pick up differences in glucose and xylose release between cultivars (Bellucci et al., 2015; Larsen, Bruun, et al., 2012; Lindedam et al., 2014). The general conclusion seems to be that among modern closely related cultivars, the differences are smaller than the analytical uncertainties. Even in one study analyzing 100 wheat cultivars spanning more than 100 years of breeding history, the conclusion was that variance in saccharification potential was poorly captured by genotypic effects (Bellucci

et al., 2015). The present study in addition provides evidence that external environmental factors such as availability of N have negligible effect on plant cell wall composition (cellulose and lignin) and recalcitrance, *that is*, saccharification potential.

#### 4.5 | From an agronomic perspective

From a biorefining perspective, the criteria for the ideal DPC are high yields and high concentration of carbohydrates that can easily be released as monomeric sugars (Townsend et al., 2017). In the present study, the hypothesis was that the N application rate would influence the yield but also cause alterations in the plant cell wall structure/composition that could influence the potential for sugar release. Based on the general response from 14 different wheat cultivars and one triticale cultivar, it can be concluded that the N application rate had a very limited influence on the composition of the plant cell wall with respect to cellulose and lignin. It was also not possible to reveal any effect on the saccharification potential, which means that the cell wall structure and nature of the cellulose were not altered in response to N application rate. However, the effects of N on the yield of both grain and straw were pronounced. Overall biomass output was increased by increasing the N application rate from 100 kg/ha to 220 kg/ha. In that perspective, the total potential for sugar release (kg glucose and xylose per ha) was therefore largely determined by the straw yield (Figure 8). The N application rate had a significant effect, but also the effect of cultivar and year was significant. Trilobit, which displayed a superior straw yield, had accordingly the overall best potential for sugar production (5.1 t of sugar per ha). Among the wheat cultivars, Creator was the best yielding cultivar with 3.7 t/ha of sugar. The worst performing cultivar was Pierrot with 2.9 t/ha. These observations are generally in line with previous studies testing the potential of different cultivars, which revealed that straw yield was a more important trait than cellulose concentration and saccharification potential/recalcitrance (Larsen, Bruun, et al., 2012).

These results point out that plant breeding for higher cellulose content and reduced recalcitrance might be difficult, as also seen in other studies (Bellucci et al., 2015). The conclusion is therefore that selection toward cultivars with higher total biomass (straw and grain) combined with optimum farming practice, *that is*, sufficient N application rate, should be the preferred pathway with respect to providing (more) biomass for biorefinery applications. In line with this, previous studies have also emphasized that a prerequisite for increasing grain yield from wheat will be to increase the total aboveground biomass (Gaju et al., 2016), which fits well with the DPC philosophy.

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#### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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